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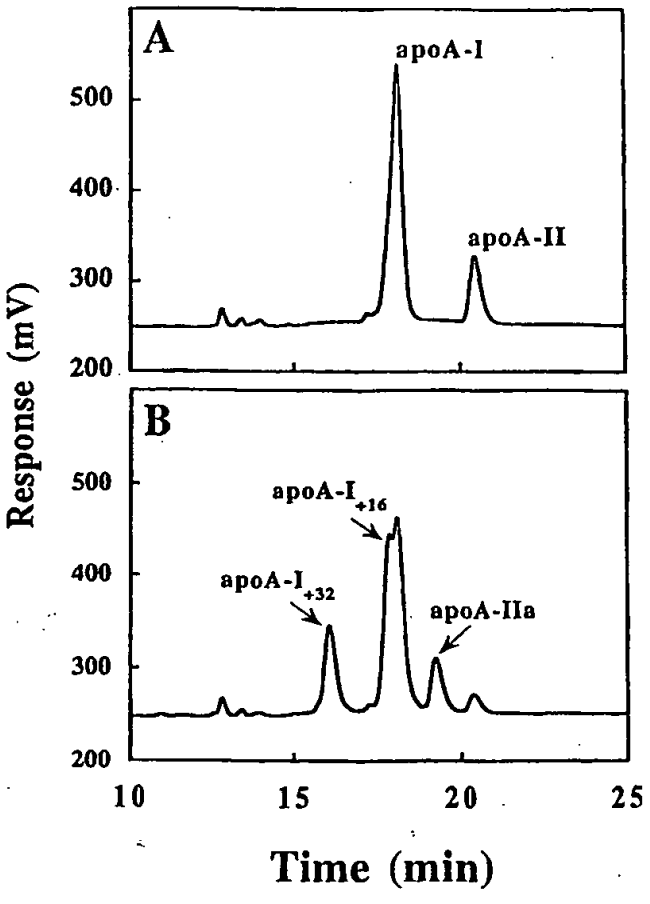
(54) Title: OXIDIZED APOLIPOPROTEINS AND METHODS OF USE



(57) Abstract: The present invention relates to oxidized forms of apolipoproteins and methods of use in diagnosis and treatment of diseases associated with oxidative stress, such as cardiovascular diseases, in particular of atherosclerosis. Specifically, oxidized forms of ApoA-I and ApoA-II having specific methionine residues oxidized are provided. Methods of use include increasing effux and α-TOH, lowering lipid concentrations and preventing or treating lipid-associated conditions are provided. Methods of detecting oxidative stress, genotypes and extent of lipid associated conditions are also provided by utilizing the oxidative apolipoproteins.

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OXIDIZED APOLIPOPROTEINS AND METHODS OF USE

The present invention relates to oxidized forms of apolipoproteins and methods of use in diagnosis and treatment of diseases associated with oxidative stress, such as cardiovascular diseases, in particular of atherosclerosis.

BACKGROUND

Oxidative damage to bio-molecules is implicated in a number of diseases, including but are not limited to cardiovascular disease such as atherosclerosis, inflammatory diseases, neurodegenerative disorders, ischemia reperfusion, etc. The presence of and elevated concentration of oxidized bio-molecules can be used for the diagnosis of disease associated with oxidative damage. The biological sample tested can be selected depending on the disease to be verified or treated.

Principally, oxidized sugars, lipids, protein and deoxyribonucleic acids (DNA) may be used for the diagnosis of oxidative damage. Of these, oxidized lipids and DNA are used most commonly while oxidized sugars are not commonly measured and assays for oxidized proteins are limited. Methods for oxidized DNA largely rely on the measurement of oxidized bases by GC/MS or HPLC with electrochemical detection. Due to their complexity, these methods are largely restricted to scientific research.

In contrast, there are commercial kits available for the determination of lipid hydroperoxides (LOOH, the primary oxidation products derived from lipids containing one or more pairs of bisallylic hydrogen atoms) and the assessment of the state of lipid peroxidation in a host. There are also several methods described for the measurement of secondary lipid oxidation, i.e. products derived from the degradation of LOOH. These include, but are not limited to, malonyldialdehyde (commonly measured as the reaction product with thiobarbituric acid, TBARS) and F2 isoprostanes (a family of non-enzymatic oxidation products derived from arachidonic acid). WO 98/12561 describes the

detection of oxidative damage in a host based on the measurement of the product formed between LOOH and lysine residues of proteins.

Methods for the detection of oxidized proteins rely on either HPLC/GC-based methods to measure specific oxidized amino acids (present after hydrolyzing the proteins of a biological sample), or the determination of the content of thiols (e.g. by a thiol-specific reagent) or carbonyls (e.g., by an antibody recognizing carbonyl groups) in intact proteins. There are no indicators or markers of diseases or oxidative damage based on the measurement of specific modification of methionine (Met) residues in known intact proteins.

High-density lipoproteins (HDL) are generally regarded as anti-atherogenic, an activity commonly attributed to the removal of extra-hepatic cholesterol by HDL particles and apolipoproteins, mainly apolipoprotein A-I (apoA-I) that dissociate from HDL. An important anti-atherogenic activity postulated to underlie the beneficial property of high HDL levels is the removal of cholesterol from peripheral tissue and its transport to the liver for excretion, a process known as reverse cholesterol transport. In addition to promoting cholesterol efflux, HDL has also been proposed to be anti-atherogenic by aiding the removal and detoxification of pro-atherogenic oxidized lipids. Thus, cholesteryl ester transfer protein transfers oxidized lipids from low-density lipoproteins (LDL) to HDL, and HDL carries the majority of cholesteryl ester hydroperoxides (CE-OOH, the first and major products formed during lipoprotein oxidation) in human plasma. HDL, but less so LDL, can reduce CE-OOH to the corresponding alcohols (CE-OH). In addition, CE-OOH and CE-OH in HDL, but not LDL, are removed rapidly via selective uptake by liver parenchymal cells *in vitro* and *in situ* perfused liver. This uptake is associated with cellular detoxification of CE-OOH and is more rapid than that of the corresponding non-oxidized cholesteryl esters (CE). Furthermore, CE-OH are also rapidly removed from HDL via hepatic clearance *in vivo*. This process is mediated by the scavenger receptor BI and associated with biliary secretion of the CE-OH-derived cholesterol, indicating a potential 'receptor'-mediated regulation of this pathway.

Once associated with HDL CE-OOH are reduced to the corresponding CE-OH that no longer contribute to or enhance the oxidative modification of lipoproteins. This 'antioxidant' activity is expressed by reconstituted HDL particles (rHDL) containing apoA-I or apoA-II only and lipid-free apoA-I and extends to phospholipid hydroperoxides. The reduction of LOOH added to or formed in HDL exposed to radical oxidants results in the formation of selectively oxidized apoA-I (*i.e.*, apoA-I_{+16} and apoA-I_{+32}) that contain one and two Met sulfoxide [Met(O)] residue(s), respectively as the sole modification. ApoA-I_{+16} and apoA-I_{+32} are respectively 16 and 32 mass units heavier than native apoA-I.

In human atherosclerotic lesions HDL is oxidized to an extent comparable to that of LDL. HDL oxidized *in vitro* has a decreased or enhanced ability to promote cellular cholesterol efflux, depending on the oxidizing conditions employed. It is therefore possible that formation of Met(O) affects the ability of apoA-I/HDL to mediate efflux of lipids from macrophages. However, until now it is not known which Met residues of apoA-I are converted to Met(O) as HDL becomes oxidized and how this affects lipid efflux.

Lipoproteins are the source of most intra- and extra-cellular lipid in atherosclerotic plaque and are present in the vessel wall at levels approximately proportional to their concentrations in plasma, but inversely related to their size (thus there is more LDL than very low density lipoprotein (VLDL) in the vessel wall). The fat that is stored in the atherosclerotic lesion comes largely from LDL which contains only one protein molecule, apolipoprotein B-100 (apoB), that threads in and out of the particle surface. ApoA-I-containing particles (HDL-derived) and apoB-containing particles (LDL- and VLDL-derived) have been extracted from human plaque, and have been identified in modified and unmodified forms.

The oxidative hypothesis of atherosclerosis argues that oxidation of LDL (and other lipoproteins) predominantly occurs in the sub-endothelial space of the vessel wall. Oxidized LDL promotes lipid accumulation in cells, can be cytotoxic to endothelial and other cells, may mediate the generation of the necrotic core, and promotes the recruitment of inflammatory cells and the expression of

adhesion molecules on endothelial cells and thrombogenic tissue factor. Accumulation of lipid by macrophages can induce the secretion of matrix metalloproteinases and cytokines (e.g. IL8). These thrombotic, adhesive and inflammatory properties of oxidized LDL may be critical for disease progression (whether episodic or continuous) and likely involves episodic damage to the endothelium.

Atherosclerosis is the predominant cause of vascular death in Westernized countries, causing myocardial infarct (MI), strokes, and peripheral vascular ischemia. Atherosclerosis involves intimal thickening and, initially at least, subendothelial deposition of lipids in arteries. At later stages, the elastic lamina separating the intima from the media no longer limits the extent of lesions, and the disease (in particular the deposition of a necrotic core) can involve the medial layer. An intramural plaque thrombosis usually precipitates acute clinical events, and this can extend intra-luminally to occlude blood flow. Such thrombosis is commonly associated with rupture of the atherosclerotic plaque, which more commonly occurs at the most cellular and structurally weakest area of advanced plaque- the shoulder region. That inflammatory cells often co-localize with sites of plaque rupture has indicated to some investigators that inflammatory markers in plasma may predict plaque rupture and acute events. The necrotic core is important in this regard also, because it is probably among the most thrombogenic components of plaque. It is important to note, however, that the pathology of acute plaque thrombosis can be variable. For example, inflammatory infiltrates and acute thrombosis can be secondary to superficial erosions of the fibrous cap without plaque rupture.

An individual advanced plaque causing a hemodynamically severe stenosis is probably more likely to cause an occlusive thrombosis than an individual hemodynamically mild lesion. However, most acute MI occur secondary to thrombosis overlying angiographically minor or moderate lesions. This may be due to a combination of factors such as the lack of collateral vessels if stenoses are hemodynamically minor, the less fibrotic (and more lipid) composition of many small plaques, and the greater abundance of minor lesions compared with severe

stenoses. Thus, many argue that the overall extent of atherosclerosis and therefore total number of lesions would be a better indicator of subsequent risk of complications from atherosclerosis than the severity of individual lesions.

The age-corrected rate of MI in Australia and other Westernized countries has decreased markedly. However, atherosclerosis is a progressive age-related disease and the average age of the population in Westernized countries has increased. As a result, the total number of heart attacks has not decreased and those suffering from ischemic heart disease are now simply older than before. Indeed, as the age of populations continues to increase, the overall incidence of atherosclerotic disease in the community can be expected to increase in the years ahead. In Asian countries including China, Singapore, and Taiwan, there are forecasts of an epidemic of coronary disease occurring in the next 10-20 years, as high tobacco smoking rates, and hyperlipidemia secondary to high saturated fated intake, are replacing traditional healthy lifestyles.

There are no accurate indicators or markers of diseases associated with oxidative stress such as cardiovascular disease which provide an insight into a patients condition. At present, the diagnosis of atherosclerosis is limited to risk assessment that does not provide direct information on the extent of disease of individuals. Understanding where the problems of lipid efflux and transport lie many provide better diagnosis of heart conditions and of cardiovascular disease.

It is an object of the present invention to overcome or at least alleviate some of the problems of the prior art.

SUMMARY OF THE INVENTION

In one aspect of the present invention there is provided an oxidized apoA-I wherein at least methionine (Met) residue 86 is oxidized to Met(O). Preferably, both Met 86 and 112 are oxidized to Met(O).

In another aspect of the invention, there is provided oxidized apolipoprotein A-II (apoA-II), wherein the apoA-II dimer is oxidized. In a preferred aspect of the invention, there is provided an oxidized apoA-II dimer wherein one Met residue at position 26 is oxidized to Met(O). In a further preferred aspect, both Met residues at position 26 of each monomer of the apoA-II dimer are oxidized.

In yet another aspect of the present invention, there is provided a method of producing specific forms of oxidized apoA-I and apoA-II. The method comprises the use of native human HDL as a source for non-oxidized apoA-I and apo-II, and oxidized HDL as a source of oxidized apoA-I and apoA-II.

In yet another aspect of the invention, there is provided a method of inducing an increased efflux of lipids from cells, said method comprising subjecting said cells to oxidized apoA-I or apoA-II oxidized at a single or several specific Met residue(s).

In yet another aspect of the present invention there is provided a method of inducing an increased efflux of α-tocopherol (α-TOH) from cells, said method comprising subjecting said cells to oxidized apoA-I or apoA-II oxidized at a single or several specific Met residue(s).

In yet another aspect of the present invention, there is provided a method of lowering lipid concentration in cells, said method comprising inducing an increased efflux of lipids from the cells by administering an effective amount of oxidized apoA-I or apoA-II oxidized at a single or several specific Met residue(s).

In yet another aspect of the present invention there is provided a method of preventing or treating lipid- associated conditions wherein said condition is associated with high levels of lipids, said method comprising administering an effective amount of oxidized apoA-I or apoA-II oxidized at a single or several specific Met residue(s).

In yet another aspect of the present invention there is provided a method of diagnosing a lipid-associated condition wherein said condition is characterized by high lipid concentration and/or other genetic factor(s) known to be associated with an increased risk of cardiovascular disease, said method comprising determining levels of apoA-I or apoA-II oxidized specifically at a single or several specific Met residue(s) and comparing against an absence of said lipid associated condition. Lipid-associated conditions may be affected by genetic, behavioral or environmental conditions including but not limited to genotypes associated with increased risk of cardiovascular disease, such as endothelial nitric oxide synthase (eNOS) genotype a/b, Asp_{298} variant of eNOS, or conditions resulting in high levels of circulating homocysteine, or life style pattern such as tobacco smoking.

In another aspect of the present invention, there is provided a method of detecting an increased risk for coronary disease, said method comprising detecting a genotype or other factor associated with an increased risk of cardiovascular disease.

Accordingly, in a preferred aspect of the present invention, there is provided a method of determining the extent of a lipid associated condition, wherein the extent of the lipid associated condition is characterized by high lipid and apolipoprotein concentration adjusted levels of an oxidized apolipoprotein A-I or A-II having an oxidation at a single or several specific Met residue(s) and comparing against an absence of said lipid associated condition.

In yet another aspect of the present invention, there is provided a method of assessing genotype, said method comprising detecting in a biological sample the presence of an oxidized apolipoprotein A-I or A-II having an oxidation at a single or several Met residue(s) and comparing against a condition where the genotype is absent.

In yet another aspect of the present invention, there is provided a method of measuring oxidative stress *in vivo* said method comprising detecting in a biological sample the presence of an increased proportion of said apolipoprotein

A-I or A-II in oxidized form having an oxidation at a single or several specific Met residue(s) and comparing against a healthy condition characterized by the absence of oxidative stress.

In another aspect of the present invention, there is provided a method of collecting biological samples for determination of oxidized apolipoproteins A-I or A-II, said method comprising the steps of:

obtaining a biological samples; and

subjecting the sample to conditions which reduce further oxidation of a single or several methionine residue(s) on the apolipoprotein.

FIGURES

Figure 1 shows HPLC traces of HDL before and after oxidation with AAPH. Typical chromatograms are shown for apolipoproteins of native HDL before (A) and directly after oxidation and removal of AAPH (B). Oxidation conditions and HPLC analyses were as described in the Examples. ApoA-I+16 and apoA-I+32 refer to oxidized forms of apoA-I that contain 1 and 2 molecules of Met(O) and are 16 and 32 mass units greater, respectively, than the native non-oxidized apolipoprotein. ApoA-IIa contains 1 molecule of Met(O) per apoA-II dimer.

Figure 2 shows HPLC traces of proteolytic peptides of apoA-I (A) and apoA-I_{+32} (B) obtained after endoproteolytic digests. 50 mg of apolipoproteins were digested using endoprotease Asp N, applied to a C18 RP-column, peptides were eluted at 1 mL/min over 30 min using a gradient of 5 to 75% acetonitrile (0.1% TFA) and monitored at 214 nm. Peaks labeled with numbers (1-10) represent or contain peptides of apoA-I or apoA-I_{+32} with identified mass and sequence (see Table I). Peaks 4 and 7 in panel A represent the Met-containing non-oxidized peptides corresponding to the Met(O)-containing oxidized peptides 4' and 7' in panel B as indicated by arrows.

Figure 3 shows increased lipid affinity of apoA-I_{+32} versus apoA-I. The clearance of DMPC multilamellar vesicles due to the addition of apoA-I or apoA-I_{+32} was

assessed by measuring the decrease in absorbance at 325 nm and 24 °C, as described in the Examples. The apparent rate constant $k_{1/2}$ was determined as the reciprocal of the time required for the optical density to reach half of its initial value. The results shown are from a single experiment typical of 9 separate apolipoprotein preparations.

Figure 4 shows enhanced efflux of [^{3}H]-cholesterol from hMDM to lipid-free apoA-I+32 versus apoA-I. Cells were isolated by elutriation and cultured as described in the Examples. Matured hMDM were then loaded with acetylated LDL (100 mg/mL) for 48 h in the presence of [^{3}H]-cholesterol (2 mCi/mL) to label cellular cholesterol pools, equilibrated, and subjected to efflux in triplicates. Cells were extracted at t = 0 min for lipid analysis by HPLC with UV 210 nm and on-line radiometric detection. Total cellular cholesterol mass and radioactivity were 153 ± 34 nmol/mg cell protein and 1 112 407 ± 102 965 cpm/mg cell protein, respectively (mean ± SD, n = 3 experiments). After equilibration cells were washed and subjected to efflux by incubation with RPMI only (control, diamonds) or RPMI supplemented with 25 mg/mL of apoA-I (circles) or apoA-I+32 (squares). At the indicated times aliquots of the medium were removed, centrifuged (to remove any cells) and the radioactivity determined in the resulting supernatant by scintillation counting. At the last time point, cells were lysed and cell-associated radioactivity and protein determined. Data shown are means ± SEM for 9 cultures obtained in 3 separate experiments. (Two-way ANOVA for apoA-I against apoA-I+32: $P<0.0001$).

Figure 5 shows efflux of phospholipids from hMDM to lipid-free apoA-I and apoA-I+32. Matured hMDM were loaded with acLDL (100 mg/mL) for 48 h, washed and labeled with [^{3}H]-methyl choline (5 mCi/mL) in the presence of 0.1 % BSA during over night incubation. Prior to the addition of efflux media cells were washed 4 times with RPMI and equilibrated for 1 h in RPMI only. This procedure resulted in 97.8 ± 19.1 nmol phospholipid or 9850334 ± 964864 cpm per mg cell protein (means ± SD), corresponding to a specific activity of 100514 cpm/nmol phospholipid. Monolayers were incubated for up to 3 h with efflux-media without

(diamonds) or with 25 mg/mL of apoA-I (circles) or apoA-I+32 (squares). Extraction of phospholipids from the media and cells and determination of radioactivity were performed as described in the Examples. Data shown are means ± SEM for 7 cultures obtained in 2 separate experiments. (Two-way ANOVA for apoA-I against apoA-I_{+32}: P = 0.05).

Figure 6 shows an efflux of α-TOH from hMDM to lipid-free apoA-I and apoA-I_{+32}. The experimental conditions were as described in the legend to Figure 4, except that cells were pre-incubated with 0.2 mCi/mL of all-rac-[^{14}C]-α-TOH during loading with acetylated LDL. After equilibration cellular α-TOH levels were 2.9 ± 0.5 nmol or 141639 ± 8124 cpm/mg cell protein (means ± SD, n=3), corresponding to a specific activity of 50130 cpm/nmol α-TOH. Monolayers were incubated for up to 3 h with efflux-media without (diamonds) or with 25 mg/mL of apoA-I (circles) or apoA-I+32 (squares). Data shown are means ± SE for 9 cultures obtained in 3 separate experiments. (Two-way ANOVA for apoA-I against apoA-I+32: P = 0.0004).

Figure 7 shows helix-wheels of the putative amphipathic helix 2 of apoA-I and apoA-I_{+32}. The models were constructed using the program PSAAM of Dr. A. Crofts, University of Illinois and the rules of Chou and Fasman as described previously by Jonas *et al.* Biochim. Biophys. Acta 1166: 202-210 (1993) for helices 4 and 6. Hydrophobic moments and angles were obtained using the Kyte/Doolittle hydropathy index. Helical sequences start with a Pro residue placed at the 0 ° position and proceed with the successive amino acid residue placed clockwise at 100 ° from the former. Empty, grey and filled circles represent hydrophobic, uncharged polar and charged residues, respectively. The positions of the Met and Met(O) residues are indicated by the arrow. To estimate the hydrophobic moments of the Met(O)-containing helices Gln was used instead of Met.

Figure 8 shows the difference in apoA-I_{+32} between early (I/II) and late (III/IV) stages of atherosclerosis in human aortas.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect of the present invention there is provided an oxidized apoA-I wherein at least Met residue 86 is oxidized to Met(O). In a preferred aspect, both Met residues 86 and 112 are oxidized to Met(O).

Preferably the oxidized apoA-I is in a form which is 16 or 32 mass units heavier than the native apoA-I and hereby designated apoA-I_{+16} or apoA-I_{+32} respectively. This form of oxidized apolipoprotein contains at least one or two Met sulfoxide [Met(O)] residue(s). ApoA-I_{+16} or apoA-I_{+32} may be *in vivo* or *in vitro* oxidized apoA-I.

In another aspect of the invention, there is provided oxidized apoA-II, wherein the apoA-II dimer is oxidized. In a preferred aspect of the invention, there is provided an oxidized apoA-II dimer wherein one Met residue at position 26 is oxidized to Met(O). In a further preferred aspect, both Met residues at position 26 of each monomer of the apoA-II dimer are oxidized.

The non-oxidized or oxidized apoAI or apoA-II may be native and derived from HDL or may derive from any source of biological material, including but not limited to, plasma, serum, blood, interstitial fluid, blood vessel(s), or any other tissue.

One method of obtaining oxidized HDL may be as follows. This is illustrative only and the application should not be limited to this method. Human HDL may be isolated rapidly from freshly obtained plasma obtained from normolipidemic donors by the two-step density gradient ultra-centrifugation method described by Sattler et al. *Methods Enzymol.* 233: 469-489 (1994). Prior to use, HDL is desalted (e.g. by size exclusion chromatography) and its protein content determined (e.g. by the bicinchoninic acid method (Sigma) using BSA (Sigma) as standard). For the isolation of non-oxidized apoA-I and apoA-II, HDL may be subjected to semi-preparative RP-HPLC, as described in detail by Garner et al. *J. Biol. Chem.* 273: 6080-6087 (1998), and the eluting fractions corresponding to

apoA-I and apoA-II collected. For the preparation of specifically oxidized apoA-I and apoA-II, isolated HDL may be oxidized in phosphate buffered saline pH 7.4 (PBS) containing 1 mM EDTA by aerobic incubation at 37 °C for 19 h in the presence of *2,2'-azobis*(2-amidinopropane) hydrochloride (e.g., AAPH, 80 micromol/L and mg HDL protein). AAPH is a generator of aqueous peroxyl radicals. After oxidation, AAPH may be removed by gel filtration and the oxidized HDL subjected to semi-preparative RP-HPLC as described above for non-oxidized apolipoproteins. The eluting fractions corresponding to the specific forms of oxidized apoA-I and oxidized apoA-II dimer may be collected. The collected fractions containing either non-oxidized or specifically oxidized apoA-I or apoA-II may be placed immediately on dry ice, freeze-dried and then dialyzed extensively against PBS (two buffer changes) and 0.5 mM Tris pH 7.4 (three changes). Protein concentrations may be determined by $A_{280\ nm}$ or by the bicinchoninic acid method described as above. These preparations of isolated apolipoproteins are free of phospholipids or other lipids as determined using commercial enzymatic kits (Boehringer Mannheim), and are stored at –80 °C until use.

The apolipoprotein may be apoA-I or apoA-II. Preferably, the apoA-I or apoA-II is derived from HDL. Many methods available to the skilled addressee may be used to isolate apoA-I or apoA-II. However HPLC is preferred. Other sources such as FPLC and immunoprecipitation. These include but are not limited to FPLC and immunoprecipitation. However HPLC is preferred to provide suitable sources of apoA-I and apoA-II.

The apolipoprotein may be oxidized by any means available to the skilled addressee. However, it is preferred that they are oxidized under controlled conditions, using 2, 2'-azobis (2-amidino propane) hydrochloride (AAPH).

In a preferred aspect of the invention, the oxidized apolipoproteins may be isolated. Preferably, HPLC or RP-HPLC is used to isolate the oxidized forms. However other methods available to the skilled addressee may be used such as

FPLC. Once the oxidized apoliproteins undergo HPLC they may be identified by their distinctive peaks for instance as shown in Figure 1, and/or by MS.

In yet another aspect of the present invention there is provided a reconstituted HDL comprising an oxidized apolipoprotein, said apolipoprotein having a single or several Met residue(s) oxidized. Preferably the apolipoprotein is apoA-I having at least the Met 86 residue oxidized. More preferably both the Met 86 and 112 residues(s) are oxidized.

In another preferred aspect, the reconstituted HDL comprises an oxidized apoA-II having at least one Met residue oxidized. Preferably the Met 26 is oxidized on at least one monomer of apoA-II. More preferably, both Met 26 residues in the apoA-II dimer are oxidized.

In yet another aspect of the invention, there is provided a method of inducing an increased efflux of lipids from cells, said method comprising subjecting said cells to an oxidized apolipoprotein wherein said apolipoprotein is oxidized at a single or several Met residue(s).

Preferably, the lipids are selected from the group including cholesterol, phospholipids, fatty acids and lipid-soluble antioxidants.

The oxidized apolipoprotein may be selected from apoA-I or apoA-II or a mixture of both.

Applicants have found that the oxidized form of apoA-I which is designated apoA-I_{+32}, has an enhanced ability to induce (above normal levels), the efflux of lipids from lipid-laden human cells. It has also been found that apoA-I_{+32} converted multilamellar liposomes to small unilamellar vesicles 2 to 3 times faster than apoA-I. Without being restricted by theory, it would suggest that the introduction of the sulphoxide moieties increases the ability of apoA-I or apoA-II to interact with phospholipids and other lipids.

It is preferred that the oxidized apolipoprotein is specifically oxidized at the Met 86 residue. More preferably both Met 86 and Met 112 residues for apoA-I_{+32} or Met 26 residue for apoA-II_{+32} are oxidized.

Preferably the efflux is increased over the level provided by non-oxidized apoA-I or apoA-II. ApoA-I promotes the efflux of cholesterol from cells and it has been shown by the applicants that the oxidized form significantly increases the efflux of cholesterol.

Preferably, the apoA-I_{+32} or oxidized apoA-II are substantially lipid-free to accept lipid from the cell and to carry the lipid.

In yet another aspect of the present invention there is provided a method of inducing an increased efflux of α-tocopherol (α-TOH) from cells, said method comprising subjecting said cells to oxidized apoA-I or apoA-II oxidized at a single or several specific Met residue(s).

Preferably, the apolipoprotein is apoA-I or apoA-II. More preferably the apoA-I is specifically oxidized at the Met 86 residue to Met(O). Preferably both the Met 86 and Met 112 residues are oxidized to Met(O) together. The apoA-II may be oxidized at the Met 26 residue on at least one apolipoprotein of apoA-II. Preferably both Met 26 residues on each monomer of the apoA-II dimer are oxidized.

In yet another aspect of the present invention, there is provided a method of lowering lipid concentration in cells, said method comprising inducing an increased efflux of lipids from the cells by administering an effective amount of oxidized apoA-I or apoA-II is oxidized at a single or several Met residue(s).

Preferably, the apolipoprotein is as hereinbefore described above.

In the methods described above, it is preferred that the cells are human peripheral cells, most preferably, they are human monocyte-derived macrophages.

In yet another aspect of the present invention there is provided a method of preventing or treating lipid- associated conditions wherein said condition is associated with high levels of lipids, said method comprising administering an effective amount of oxidized apoA-I or apoA-II, oxidized at a single or several Met residue(s).

Preferably, the apolipoprotein is as hereinbefore described above.

Lipids, may be any one or more of cholesterol, phospholipids or liposomes. Without being restricted by theory, it has been proposed that the presence of the oxidized apolipoprotein increases the efflux of the lipids from cells, thereby reducing the lipid levels in those cells.

A "lipid-associated condition" as used herein is a condition when the lipid contributes to a condition which may have a positive or negative effect to the body.

Preferably the lipid-associated condition is selected from the group including but not limited to coronary vascular disease, ischemic heart disease, atherosclerosis, dyslipidemias, etc. HDL are generally regarded as anti-atherogenic and this is commonly attributed to the removal of extra hepatic cholesterol by HDL particles and apolipoproteins that dissociate from HDL. As well as promoting cholesterol efflux, HDL and apolipoproteins have also aided in the removal and detoxification of pro-atherogenic oxidized lipids thereby contributing to their anti-atherogenic properties.

The term "high levels of lipids" when applied to the lipid-associated condition is determined by comparing lipid levels in the absence of the lipid-associated

disease. This is determined by the absence of symptoms caused by abnormal levels of lipids.

Hence, in a preferred aspect, the invention further provides a method of preventing and treating atherosclerosis, said method comprising administering an effective amount of an oxidized apolipoprotein, wherein said apolipoprotein is oxidized at a single or several methionine residue(s).

In the methods described above, the apolipoprotein may be administered in an oxidized form either alone or as a reconstituted HDL as hereinbefore described.

The term "effective amount" as used herein means an amount that increases the efflux of lipids from cells above that provided by non-oxidized apolipoproteins.

Preferably, the oxidized apolipoprotein is as hereinbefore described above.

Methods of detecting the oxidized forms of the apolipoprotein may be any method available to the skilled addressee such as, but not limited to, HPLC, FPLC, or methods using ligands/agents which bind to the oxidized forms of apoA-I or apoA-II, such as, for example, antibodies to the oxidized forms of apoA-I or apoA-II. Antibodies available for non-oxidized apoA-I and/or apoA-II may also be used as they can cross-react with oxidized apoA-I and apoA-II, providing suitable controls are performed to normalize the results. Commercial antibodies to apoA-I or apoA-II may include, but are not limited to Biodesign monoclonal antibodies H45615M, H45625M or H61531M.

Various known forms of immunoassays may be used for detecting and/or measuring oxidized apoA-I or apoA-II, such as for example EliSA, RIA, IRMA and the like. Similar assay formats may be employed with other ligands which bind to oxidized apoA-I or ApoA-II".

Determination of a condition may be made by the comparison of a normal to an abnormal level of oxidized apolipoprotein.

In yet another aspect of the present invention, there is provided a method of assessing genotype, said method comprising detecting in a biological sample the presence of an oxidized apoA-I or apoA-II having an oxidation at a single or several Met residue(s) and comparing against a condition where the genotype is absent or different.

Preferably the oxidized apolipoprotein(s) is/are as described above.

The genotype and the level of oxidized apolipoprotein may be correlated. Preferably the genotype assessed is a polymorphism of a genotype associated with an increased risk for coronary disease. It is preferred that the genotype is the eNOS a/b genotype. It has been found by the applicants that there is a positive correlation of this genotype and percentage of apolipoprotein A-I present as apoA-I_{+32}. The eNOS a/b genotype has a different level of oxidized apolipoprotein to the common eNOS b/b genotype and at times the eNOS a/b genotype may have approximately 5 times higher oxidized apolipoproteins than the eNOS b/b genotype. Therefore by measuring this correlation, genotypes may be predicted and risk of coronary disease further assessed.

In yet another aspect of the present invention, there is provided a method of diagnosing a lipid associated condition wherein said condition is characterized by high lipid concentration and/or genetic factors known to be associated with an increased risk of cardiovascular disease, said method comprising determining levels of an apoA-I or apoA-II oxidized at a single or several Met residue(s) and comparing against levels in an absence of said lipid associated condition.

The genetic conditions include, but are not limited to, genotypes associated with increased risk of cardiovascular disease, such as endothelial nitric oxide synthase (eNOS) genotype a/b, Asp_{298} variant of eNOS, or conditions resulting in high levels of circulating homocysteine.

In another aspect of the present invention, there is provided a method of detecting an increased risk for coronary disease, said method comprising detecting a genotype associated with an increased risk of cardiovascular disease.

Preferably, the genotype is an eNOS a/b genotype which is associated with an increased risk of coronary disease. The common eNOS b/b genotype is less likely to correlate with coronary disease.

Accordingly, in a further aspect of the present invention, there is provided a method of determining the extent of a lipid-associated condition wherein said extent of the lipid associated condition is characterized by a high lipid and/or apolipoprotein concentration adjusted level of an oxidized apolipoprotein having an oxidation at a single or several specific Met residue(s) and comparing against an absence of the lipid-associated condition.

In this regard, the presence of oxidized apolipoprotein, as hereinbefore described, may be used as a monitor for patients which may have a predisposition for a lipid associated condition.

The lipid-associated condition may be as described above. Preferably, the condition is atherosclerosis.

Risk factors, such as an elevated serum cholesterol concentration, contribute to the estimation of overall risk of developing atherosclerotic disease in one population relative to another population without (or with less of) the risk factor. A biochemical marker, such as an oxidized apolipoprotein, as hereinbefore described, arises from the disease itself, and, unlike a risk factor, will assess the extent and nature of disease in individuals. Hence the "extent" of the lipid associated disease or of atherosclerosis, is quite distinct from assessing a risk or lipid-associated disease.

There are many known risk factors for the development of atherosclerosis in general and coronary disease in particular. For coronary disease, LDL

cholesterol, HDL cholesterol, tobacco smoking, hypertension, inactivity, abdominal obesity, family history and diabetes are all well-established risk factors with additive (some argue multiplicative) risk for eventual coronary disease. However, there are many cases of coronary disease where there is no clear risk conferred by known risk factors, and, although Lp(a), homocysteine and other factors are being considered, the ability to predict who will develop coronary disease is at best very crude. The inadequacy of conventional risk factors is reinforced by the so-called "paradox" of French and Swiss populations who demonstrate much lower incidences of coronary disease than would be expected from their serum cholesterol level. Within populations demonstrating commonly observed cholesterol concentrations (e.g. 5.0-6.5 mM) the ability of cholesterol concentration to predict coronary disease incidence is even less reliable. In summary, for a particular individual, a good risk factor profile does not guarantee against coronary disease, and an unfavorable profile does not predict coronary disease. This becomes a very important issue in the targeting of populations for treatment of risk factors.

Targeting of populations for risk factor treatment is increasingly important in Westernized countries as well. An example of a population for whom the benefits of treatment are uncertain is that of postmenopausal women. Cholesterol is not as powerful a predictor of coronary disease in women as in men, yet women suffer an increasing proportion of heart disease in the community. A biochemical test quantifying atherosclerosis would allow recommendation of lipid-lowering therapy and/or hormone replacement therapy in those women with evident, sub-clinical atherosclerosis, and could even potentially be used to assess the effects of treatment.

Another group who would immediately find use for such a marker preferably the oxidized apolipoprotein(s) as hereinbefore described would be premenopausal female members of families with a strong family history of coronary disease, such as those with heterozygous familial hypercholesterolemia, but who have only moderate cholesterol levels and who are just entering reproductive years. If the disease is absent by biochemical assay, lipid lowering could be deferred until

after childbearing and perhaps even until after the menopause as long as their monitored assay stayed normal. This would potentially decrease their requirement for drug therapy for 20-30 years.

In yet another aspect of the present invention, here is provided a method of measuring oxidative stress *in vivo* said method comprising detecting the presence of an oxidized apolipoprotein having an oxidation at a single or several Met residue(s) and comparing against a condition in the absence of oxidative stress.

In another aspect of the present invention, there is provided a method of collecting biological samples for determination of oxidized apolipoproteins, said method comprising the steps of:

obtaining a biological samples; and

subjecting the sample to conditions which reduce further oxidation of a single or several methionine residue(s) on the apolipoprotein.

It has been found by the applicants that apolipoproteins spontaneously oxidize in collected lipid-containing samples and it has been observed that the amount of oxidized apolipoprotein increases over time. Hence it is imperative that biological samples are treated immediately, upon collection to reduce further oxidation to provide an accurate picture of the level of oxidized apolipoproteins in biological samples. Preferably, within 10 minutes of collection, samples are treated to reduce further oxidation and then stored at –70°C.

The biological sample may be any sample selected from the group, but not limited to, plasma, serum, blood, interstitial fluid, blood vessel(s), saliva or any other tissue.

In conditions which reduce further oxidation of a single or several Met residue(s) may be any condition which generally reduces oxidation of lipids and proteins or specifically targets the Met residues. Another condition that reduces further oxidation of a single or several Met residues of apoA-I or apoA-II is the

dissociation of these apolipoproteins from lipids as can be achieved e.g. by sodium dodecyl sulfate.

"Biological samples may thus be treated, at the time of collection or shortly thereafter, with for example SDS or another agent that it capable of dissociating apolipoprotien from lipid".

Adherence to this protocol should reduce the amount of oxidation so that the degree of oxidation measured accurately reflects that present in the biological sample.

The present invention will now be more fully described with reference to the following examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLES

Experimental Methods

(a) *Isolation and selective oxidation of HDL*---Human HDL was isolated rapidly by two-step density gradient ultracentrifugation of freshly obtained plasma obtained from normolipidemic donors (Sattler et al. (1994) *Methods Enzymol.* 233: 469-489). Prior to use, HDL is desalted e.g. by size exclusion chromatography (PD-10 column, Pharmacia) and its protein content determined by the bicinchoninic acid method (Sigma) using BSA (Sigma) as standard. Isolated HDL is oxidized in phosphate buffered saline pH 7.4 (PBS) containing 1mM EDTA by aerobic incubation at 37 °C for 19 h in the presence of *2,2'-azobis*(2-amidinopropane) hydrochloride (AAPH, 80 micromol/L and mg HDL protein), a generator of aqueous peroxyl radicals. After oxidation, AAPH was removed by gel filtration (Garner et al. *J. Biol. Chem.* **273,** 6080-6087 (1998)).

(b) *Isolation of native and selectively oxidized apoA-I*---ApoA-I and apoA-I_{+32} were isolated by semi-preparative RP-HPLC, as described previously. Peaks corresponding to apoA-I and apoA-I_{+32} were collected, placed immediately on dry ice, freeze-dried and then dialyzed extensively against PBS (two buffer changes) and 0.5 mM Tris pH 7.4 (three changes). Protein concentrations were determined by $A_{280\ nm}$ or by the bicinchoninic acid method described as above. Preparations were examined for their content of phospholipids using an enzymatic kit (Boehringer Mannheim).

(c) *Endoprotease Digest*---ApoA-I and apoA-I_{+32} (~50 mg in ~200 mL) were digested at 37 °C for 20 h using endoprotease Asp N (sequencing grade, Boehringer Mannheim) in 400 mL of 100 mM ammonium bicarbonate pH 8.0 at an enzyme to substrate ratio of 1:100 (w/w). The pH of each digest was then lowered to ~2.0 with 1% trifluoroacetic acid and the mixture applied to a C18 RP-column (5 mm, 300 Å, 4.6 x 250 mm, Separations Group, Hesperia, CA, USA). Peptides were eluted at 1 mL/min over 30 min using a gradient of 5 to 75% acetonitrile containing 0.1% trifluoroacetic acid. Peaks with major absorbance at 214 nm were collected, lyophilized and then dissolved in acetonitrile/water/0.05% trifluoroacetic acid (~50 mL) for mass determination.

(d) *Mass Spectrometry*---Electrospray ionization mass spectra were acquired using a single quadrupole mass spectrometer equipped with an electrospray ionization source (Platform, VG-Fisons Instruments, Manchester, UK). Samples (~50 pmol, 10 mL) were injected into a moving solvent (10 mL/min; 50:50 water:acetonitrile containing 0.1% trifluoroacetic acid) coupled directly to the ionization source via PEEK tubing (127 mm x 40 cm). The source temperature was 50 °C and N_2 was used as nebuliser and drying gas. Sample droplets were ionized at a positive potential of ~3 kV and transferred to the mass analyzer with a cone voltage of 50 V. The peak width at half height was 1 Da. Spectra of proteins were acquired in multi-channel acquisition mode over the mass range of 700 to 1800 Da in 5 sec then calibrated with horse heart myoglobin (Sigma). Spectra of peptides were also acquired in multi-channel acquisition mode over the mass range 250 to 2000 Da in 10 sec. Some spectra were recorded using an HP

LC/MSD 1100 mass spectrometer (Hewlett Packard, Palo Alto, CA, USA) employing similar conditions.

(e) *Automated N-Terminal Sequencing*---Peptides (typically 50-200 pmol) were N-terminally sequenced using an Applied Biosystems model 470A automated protein sequencer (Applied Biosystems).

(f) *Preparation and characterization of discoidal reconstituted HDL (rHDL)*---rHDL discs containing either apoA-I (rHDLA-I) or apoA-I+32 (rHDLA-I+32) were prepared by cholate dialysis (Rye, K. A., and Barter, P. J. (1994) *J. Biol. Chem.* **269,** 10298-10303) using the respective purified apolipoproteins. Compositional analyses were performed on a Cobas autoanalyzer (Roche Diagnostics, Switzerland). The Stokes' diameter and surface charge of the particles were determined by non-denaturing polyacrylamide gradient and agarose gel electrophoreses, respectively (Rye, K. A., and Barter, P. J. (1994) *J. Biol. Chem.* **269,** 10298-10303).

(g) *Biophysical characterization*---The secondary structure of apolipoproteins was analyzed by circular dichroism. Spectra of apoA-I or apoA-I+32 (0.1 mg/mL 0.5 mM Tris, pH 7.4) and $rHDL_{A\text{-}I}$ or $rHDL_{A\text{-}I+32}$ (0.16 mg of protein/mL 1 mM PBS pH 7.4) were recorded on a JASCO 720 CD spectropolarimeter at 25 °C using a 1 and 0.1 mm path length cell, respectively. The acquisition parameters were: range = 184-260 nm; resolution = 0.5 nm; band width = 1.0 nm; response time = 1 sec; scan speed = 20 nm/min, and number of scans = 16. The mean residue ellipticity was calculated by $q_{MRW} = q_{obs} \times MRW / 10 \times c \times l$, where q_{obs} is the observed raw data in millidegrees (mdg), MRW is the mean residue weight (i.e. 115.0 for apoA-I), c is the concentration and l is the path length of the cell. The secondary structure was predicted by analyzing the CD spectra using the program 'variable selection' (VARSELEC), where fitting of a database of 33 proteins of known secondary structures is performed (Compton, L. A., and Johnson, W. C., Jr. (1986) *Anal. Biochem.* **155,** 155-167 and Manavalan, P., and Johnson, W. C., Jr. (1987) *Anal. Biochem.* **167,** 76-85).

The lipid affinity of apoA-I and apoA-I+32 was determined using the DMPC clearance assay (Pownall, H. J., Pao, Q., Hickson, D., Sparrow, J. T., Kusserow, S. K., and Massey, J. B. (1981) *Biochemistry* **20,** 6630-6635). Briefly, the decrease in turbidity of multilamelar DMPC vesicles (0.5 mg/mL) upon addition of apoA-I and apoA-I_{+32} (0.2 mg/mL) was measured over time at 325 nm and 24 ± 0.2 °C using a UV/VIS Lambda 40 spectrophotometer (Perkin Elmer).

(i) *Cell culture*---Monocytes were isolated by counter-flow centrifugal elutriation (Thomas, S. R., Mohr, D., and Stocker, R. (1994) *J. Biol. Chem.* **269,** 14457-14464) from buffy coats prepared from blood from normolipidemic volunteers. Cells were plated in 12-well tissue culture plates (Falcon) at a density of $1.5x10^6$ cells per mL RPMI medium containing 10 % heat-inactivated human serum and left to adhere and differentiate into macrophages (hMDM) over the next 10 days. Subsequently, cells were washed and incubated in RPMI medium containing 10 % lipoprotein depleted serum in the presence of 100 mg/mL of acetylated LDL (acLDL) for 48 h to generate lipid-laden 'foam cells' as described (Kritharides, L., Christian, A., Stoudt, G., Morel, D., and Rothblat, G. H. (1998) *Arterioscler. Thromb. Vasc. Biol.* **18,** 1589-1599). For metabolic labeling of cellular cholesterol pools, acLDL was first labeled with [^{3}H]-cholesterol (51 Ci/mmol, Amersham) for 6 h (Kritharides, L., Christian, A., Stoudt, G., Morel, D., and Rothblat, G. H. (1998) *Arterioscler. Thromb. Vasc. Biol.* **18,** 1589-1599), before [^{3}H]-cholesterol-acLDL was diluted in RPMI to a final concentration of 100 mg/mL acLDL and 2 mCi/mL [^{3}H]-cholesterol and then incubated with cells. The 'labeling' medium was then replaced with RPMI medium without serum for 18 h to equilibrate labeled cholesterol among cellular pools (Jian, B., de la Llera-Moya, M., Ji, Y., Wang, N., Phillips, M. C., Swaney, J. B., Tall, A. R., and Rothblat, G. H. (1998) *J. Biol. Chem.* **273,** 5599-5606). The same procedure was used for metabolic labeling of cellular α–TOH pools, using 0.2 mCi/mL of all-rac-[^{14}C]-a-tocopherol (a generous gift of Eisai Co. Ltd, Japan 56) instead of [^{3}H]-cholesterol. To label phospholipids, hMDM were incubated with acLDL as described above and labeled during the 18 h equilibration period with 5 mCi/mL of methyl-[^{3}H]-choline chloride (Amersham) in the presence of 0.1% BSA (Francis, G.A., Knopp,

R. H., and Oram, J. F. (1995) *J. Clin. Invest.* **96,** 78-87). Subsequently, cells were washed twice with RPMI, incubated with fresh medium for 1 h and washed twice with RPMI containing 0.1% BSA, and then twice with RPMI before efflux incubations.

(j) *Lipid efflux studies*---After the appropriate labeling protocol, cells were incubated at 37 °C with 2 mL RPMI per dish and 25 mg per mL of either lipid-free or lipid-associated apoA-I or apoA-I_{+32} as indicated for individual experiments. In previous studies, 25 mg of apoA-I has been shown to be saturating for cholesterol efflux from primary macrophages (Kritharides, L., Jessup, W., Mander, E. L., and Dean, R. T. (1995) *Arterioscler. Thromb. Vasc. Biol.* **15,** 276-289). To investigate initial kinetics, efflux experiments were performed as described previously (Johnson, W. J., Chacko, G. K., Phillips, M. C., and Rothblat, G. H. (1990) *J. Biol. Chem.* **265,** 5546-5553). At the indicated times, aliquots of efflux media were collected, centrifuged to remove cell debris and the radioactivity determined by counting. Where indicated, the label remaining within the cells was also determined. For this, cells were washed twice with PBS containing 0.1 % (w/v) BSA, twice with PBS, lysed in 0.2 N NaOH for 10 min on ice, and aliquots of the lysates then used to count radioactivity and to determine protein using the BCA assay. After equilibration and before efflux (*i.e.*, time 0), cells in separate dishes were lysed, the lipids extracted with methanol/hexane (1:5; v:v) and then analyzed by HPLC with UV_{210nm} (for cholesterol and cholesterol esters), electrochemical or fluorescent (for α-TOH) and on-line radiometric detection (for [^{14}C]-a-TOH, [^{3}H]-cholesterol and [^{3}H]-cholesterol esters) (Christison, J., Rye, K.-A., and Stocker (1995) *J. Lipid Res.* **36,** 2017-2026 and Witting, P.K., Mohr, D., and Stocker, R. (1999) *Methods Enzymol.* **299,** 362-375). In some experiments, lipids in the efflux media were also extracted and analyzed as above. For experiments with methyl-[^{3}H]-choline-labeled cells, media and cells were stored frozen (-80 °C) until protein determination and extraction of phospholipids with 2 x 1 mL hexane/isopropanol (3:2; v:v) (18 and 1 h for the first and second extraction, respectively). The combined organic supernatants were then dried under nitrogen and extracted further using the method of Bligh and Dyer Witting, P.K., Mohr, D., and Stocker, R. (1999) *Methods Enzymol.* **299,** 362-375. The resulting

CH_3Cl fraction (1 vol) was back-washed four times with 1 vol methanol:H_2O (1:1) to remove any remaining radiolabeled choline, and the radioactivity in the washed CH_3Cl fraction counted. Cellular phospholipid mass at time 0 was determined as described above using an enzymatic kit (Boehringer Mannheim) instead off radioactivity counting.

Example 1 - Characterization of native and selectively oxidized apoA-I

To assess HDL protein oxidation and to isolate pure native and oxidized apoA-I, we adapted the HPLC method previously described (Garner, B., Witting, P. K., Waldeck, A. R., Christison, J. K., Raftery, M., and Stocker, R. (1998) *J. Biol. Chem.* **273,** 6080-6087; Garner, B., Waldeck, A. R., Witting, P. K., Rye, K.-A., and Stocker, R. (1998) *J. Biol. Chem.* **273,** 6088-6095; and von Eckardstein, A., Walter, M., Holz, H., Benninghoven, A., and Assmann, G. (1991) *J. Lipid Res.* **32,** 1465-1476). Fig. 1A shows a representative chromatogram of apolipoproteins in freshly isolated native HDL. ApoA-I and apoA-II were the major proteins and oxidized forms of apoA-I or apoA-II were not detected. Fig. 1b shows a representative chromatogram of the same HDL after oxidation with AAPH as described in the 'Experimental Methods' section. As can be seen, the content of both apoA-I and apoA-II was decreased with the concomitant formation of new peaks. The two apoA-I derived peaks have been shown previously to correspond to modified apoA-I containing one and two Met(O) instead of Met (Garner, B., Witting, P. K., Waldeck, A. R., Christison, J. K., Raftery, M., and Stocker, R. (1998) *J. Biol. Chem.* **273,** 6080-6087; Garner, B., Waldeck, A. R., Witting, P. K., Rye, K.-A., and Stocker, R. (1998) *J. Biol. Chem.* **273,** 6088-6095; and von Eckardstein, A., Walter, M., Holz, H., Benninghoven, A., and Assmann, G. (1991) *J. Lipid Res.* **32,** 1465-1476). However, these previous studies did not establish which Met residues were modified in AAPH-oxidized HDL. In this study, the mass of collected apoA-I_{+32} was determined to be 28,112.5 ± 2 Da (mean ± SD, n = 10) (Table I). This is 33 ± 2 mass units greater than that obtained for apoA-I (28,079.1 ± 1 Da, n = 10; the value predicted from the amino acid composition of apoA-I is 28,078.7 Da), consistent with the mass difference of 32 units determined previously (Garner, B., Witting, P. K., Waldeck, A. R., Christison, J. K., Raftery, M., and Stocker, R. (1998) *J. Biol. Chem.* **273,** 6080-6087; and

Garner, B., Waldeck, A. R., Witting, P. K., Rye, K.-A and Stocker, R. (1998) J. Biol, Chem. 273, 6088-6095).

Table I. Characterization of lipid-free and lipid-associated apoA-I and apoA-I+32.

	Protein	Composition (molar ratio) Phospholipid	Cholesterol	Mass (Da)	Migration distance ($mm.s^{-1}/V.cm^{-1}$)	Stokes diameter (nm)
ApoA-I	1	= 0.07	n.d.	28079.1 ± 1	-0.43	
ApoA-I_{+32}	1	= 0.10	n.d.	28112.5 ± 2	n.d.[a]	
rHDLA-I	1	91	12	n.d.	-0.46	10.0
rHDLA-I_{+32}	1	90	11	n.d.	-0.46	10.0

Discoidal HDL reconstituted with apoA-I or apoA-I_{+32} were prepared as described in 'Experimental Methods'. The characterization of 'lipid-free' and lipid-associated apoA-I and apoA-I+32 was carried out as described under 'Experimental Methods'. The stoichiometric variation in the molar ratio of protein / phospholipid / cholesterol of the particles was < 5 % between preparations.

[a]not determined.

To identify the Met residues oxidized in apoA-I_{+32}, AspN digests of purified apoA-I and apoA-I_{+32} were subjected to RP-HPLC (Fig. 2) and the mass of individual collected peptides determined by Electrospray ionization-mass spectrometry (ESI-MS). Among the peptides isolated from apoA-I, peptides 73-88, 102-127, and 128-149 (corresponding to peaks 4, 6 and 7, respectively in Fig. 2A) contained Met as verified by *N*-terminal sequencing (Table II). The corresponding peptides 73-88 and 102-127 derived from apoA-I_{+32} eluted with slightly shorter retention times (peaks 4' and 7', respectively in Fig. 2B) and their masses were exactly 16 mass units heavier when compared to their counterparts derived from apoA-I (Table II). By contrast, peptide 128-149 eluted at 16.8 min (peak 6 in Fig. 2) and its mass was 2579 (Table II), independent of whether it was derived from apoA-I or apoA-I_{+32}. This demonstrates that Met^{86} and Met^{112} (but not Met^{148}) are oxidized in apoA-I_{+32}.

Table II. ESI-Mass spectrometry and amino acid sequences of isolated peptides produced by endoproteolytic digests of apoA-I and apoA-I_{+32}.

Peptide Peak	Residues	Sequence[a]	ApoA-I $M_{r(calc)}$[b]	ApoA-I $M_{r(meas)}$[c]	ApoA-I_{+32} $M_{r(meas)}$[c]
1	150-156		823.9	824.4	824.5
2	234-243		1253.4	1253.8	1253.8
3	157-167		1243.4	1244.0	1243.8
4, 4'	73-88*	DNLEKETEGLRQE**M**SK	1907.1	1907.3	1923.5
5	89-101		1531.8	1532.1	1532.4
6	128-149*	EGARQKLHELQEKLSPLGEE**M**R	2578.9	2579.7	2579.1
7, 7'	102-127*	DDFQKKWQEE**M**ELYRQKVEPLRAELQ	3337.8	3338.1	3354.4
8	28-47		2223.5	2224.1	2224.4
9	223-233		1227.4	1228.0	1227.8
10	51-72		2555.8	2556.6	2556.9

Apolipoproteins isolated from RP-HPLC were digested with endoprotease AspN. The resulting peptides were then separated by RP-HPLC (see Figure 2), collected, lyophilized, and subjected to electrospray ionization-mass spectrometry and automated N-terminal amino acid sequencing. Peptides shown are numbered according to the peak labels shown in Figure 2. **M** in bold indicates Met residues identified to be modified in apoA-I_{+32}-derived peptides.

$M_{r(calc)}$, relative mass (Da) calculated.

$M_{r(meas)}$, relative mass (Da) measured.

*indicates peptides containing one Met or Met(O).

Isolated apoA-I and apoA-I_{+32} were essentially devoid of phospholipids (Table I). Also, the composition and properties of $rHDL_{A-I}$ and $rHDL_{A-I+32}$ were indistinguishable with regards to the molar ratio of

protein/phospholipid/cholesterol, electrophoretic mobility and particle size (Table I).

As a change in electrophoretic migration of rHDL is indicative of alteration of the secondary and/or tertiary structure of apoA-I (Sparks, D. L., Lund-Katz, S., and Phillips, M. C. (1992) *J. Biol. Chem.* **267,** 25839-25847; and Sparks, D. L., Phillips, M. C., and Lund-Katz, S. (1992) *J. Biol. Chem.* **267,** 25830-25838) the results indicate that introduction of two Met(O) does not result in a major conformational change of lipid-associated apoA-I.

CD measurements were performed to examine the secondary structure of lipid-free and lipid-associated apoA-I and apoA-I_{+32}, using the fitting procedure given in Table III. In agreement with a previous report (Jonas, A., Wald, J. H., Toohill, K. L., Krul, E. S., and Kezdy, K. E. (1990) *J. Biol. Chem.* **265,** 22123-22129), a-helix was the predominant structural type of apoA-I, accounting for 52.0 ± 2.5 % of the protein. The corresponding value for apoA-I_{+32} was the same (Table III). As for the lipid-free proteins, almost identical a-helical contents were obtained for $rHDL_{A-I}$ and $rHDL_{A-I+32}$, although the values were higher (*i.e.*, 69 and 68 %, respectively), again in agreement with literature data Jonas, A., Wald, J. H., Toohill, K. L., Krul, E. S., and Kezdy, K. E. (1990) *J. Biol. Chem.* **265,** 22123-22129. In contrast, significant albeit small differences were observed for the lipid-free apolipoprotein when comparing parallel b-sheet (4.3 ± 1.1 and 1.7 ± 1.5 for apoA-I and apoA-I_{+32}, respectively) and b-turns (16.0 ± 1.7 versus 20.7 ± 1.5); there was no significant difference in the content of anti-parallel b-sheet. However, when associated with phospholipids, these differences were no longer apparent (Table III).

Table III. Secondary structure prediction of lipid-free and lipid-associated apoA-I or apoA-I_{+32}.

	H[a]	A	P	T	O	Total
ApoA-I[b]	52.0 ± 2.5	3.6 ± 2.8	4.3 ± 1.1	16.0 ± 1.7	21.3 ± 0.5	98 ± 1.0
ApoA-I+32[b]	52.6 ± 2.5	2.6 ± 3.0	1.7 ± 1.5*	20.7 ± 1.5*	22.0 ± 1.0	100 ± 3.0
rHDL$_{A-I}$	69.0	1.0	5.0	13.0	12.0	100
rHDL$_{A-I+32}$	68.0	1.0	4.0	13.0	14.0	100

CD spectra were recorded on a JASCO spectrometer between 184 and 260 nm as described under 'Experimental Procedures'. Analysis of the secondary structure was performed using the 'variable selection' procedure described in 'Experimental Procedures'.

[a]Secondary structures (%): H, a-helix; A, anti-parallel b-sheet; P, parallel b-sheet; T, b-turn; O, 'other' structures.

[b]Data shown represent means ± SD of 3 separate protein preparations.

*Significantly different from corresponding value for apoA-I (P = 0.02).

Example 2 - Biochemical Characterization

As the ability to associate with lipids is a feature determining the ability of apoA-I to promote cellular lipid efflux, the rate of clearance of multilamelar DMPC liposomes by lipid-free apoA-I and apoA-I_{+32} was examined. As can be seen in Figure 3, the kinetics of liposome clearance were similar, although the time required for the initial relative turbidity [(A_0 – $A_\bullet$) / A_0] to decrease to half (*i.e.*, $t_{1/2}$) was significantly shorter for apoA-I_{+32} than apoA-I. Thus, the rate constant, $k_{1/2}$ ($k_{1/2} = 1/t_{1/2}$) (28) was 0.7 ± 0.4 and 1.6 ± 0.8 min^{-1} for apoA-I and apoA-I_{+32}, respectively (mean ± SD, n = 9; P = 0.0003). The resulting ratio of $k_{1/2}^{A\text{-}I+32}$ /

$k_{1/2}^{A-I}$ was 2.4 ± 0.5 (Fig. 3), indicating that apoA-I_{+32} converted multilamellar multilamelar liposomes to small unilamellar unilamelar vesicles 2 to 3 times faster than apoA-I. This suggests that the introduction of the sulfoxide moieties increased the ability of apoA-I to interact with phospholipids.

Example 3 - Cellular Studies

ApoA-I promotes the efflux of cholesterol from peripheral cells (Oram, J. F., and Yokoyama, S. (1996) *J. Lipid Res.* **37**, 2473-2491) and α-helices containing Met^{86} and Met^{112} are thought to be important for this process (Segrest, J. P., Jones, M. K., De Loof, H., Brouillette, C. G., Venkatachalapathi, Y. V., and Anantharamaiah, G. M. (1992) *J. Lipid Res.* **33**, 141-166; and Banka, C. L., Black, A. S., and Curtiss, L. K. (1994) *J. Biol. Chem.* **269**(14), 10288-10297). We therefore tested whether selective oxidation of apoA-I altered cholesterol efflux from lipid-laden hMDM. For this we pre-incubated the cells for 48 h with medium containing acLDL and [^{3}H]-cholesterol and then overnight with medium without the supplements (see Methods). During such lipid-loading and subsequent equilibration, cells acquired 6.6 % of the radioactivity added (*i.e.*, 1 112 407 ± 102 965 cpm/mg cell protein; mean ± SD). Cellular [^{3}H]-cholesterol and cholesterol mass were distributed equally between unesterified and esterified cholesterol pools as determined by HPLC with UV_{210nm} and on-line radiometric detection (not shown). Three independent experiments showed that 51 - 73 % of cholesterol label and mass were present as cholesteryl esters. After a 3 h incubation with 25 mg/mL of lipid-free apoA-I or apoA-I_{+32}, 4.6 ± 0.4 or 6.9 ± 0.6 %, respectively of the cellular [^{3}H]-cholesterol was released into the medium. In corresponding control incubations (medium alone) only 1.4 ± 0.2 % of the cellular radioactivity was released. The enhanced efflux of [^{3}H]-cholesterol to apoA-I_{+32} remained significant throughout the time course studied (Figure 4).

Cells contain several distinct pools of cholesterol, the efflux of which may rely on different mechanisms characterized by different kinetics. We therefore tested the efflux of cholesterol from hMDM to lipid-free apoA-I and apoA-I_{+32} after long-term incubations up of to 24 h. Efflux of [^{3}H]-cholesterol was linear throughout this period of time (data not shown). As compared to control (medium only), the

presence of apoA-I and apoA-I+32 promoted efflux of [^{3}H]-cholesterol 2.3- and 2.8-fold, respectively (Table IV). After 24 hours, cholesterol efflux was significantly greater for apoA-I+32 than apoA-I, but only by a factor of 1.2. This and the results from the short-term incubation suggest that the enhanced ability of selectively oxidized, lipid-free apoA-I to remove cellular cholesterol is observed primarily during the initial stages of incubation of the apolipoprotein with the cells. This is consistent with the enhanced lipid affinity observed for apoA-I_{+32} versus apoA-I (Figure 3).

Table IV. Efflux of cholesterol from hMDM during long-term incubation with lipid-free and lipid-associated apoA-I and apoA-I_{+32}.

	% Total [^{3}H]-Cholesterol	T-Test[a]
RPMI medium (control)	8.3 ± 0.7	
ApoA-I	19.0 ± 1.3	
ApoA-I_{+32}	22.9 ± 1.6	P = 0.007

Human MDM foam cells were metabolically labeled with [^{3}H]-cholesterol, and efflux studies performed for 24 h in RPMI medium in the absence (control) or presence of 25 ?g/mL of the acceptor protein indicated, as described under 'Experimental Procedures'. Following incubation, the [^{3}H] activity was determined in the medium and cell lysates. Cholesterol efflux is expressed as the percentage of the total [^{3}H] activity (medium plus cells) and normalized to cellular protein. The data shown are means ± SE for 12 or 4 cultures obtained from 4 separate experiments for lipid-free apoA-I and apoA-I_{+32}, respectively. Cholesterol efflux was significantly higher in the presence of apolipoprotein acceptor than medium alone.

[a]P-value is for comparison with the corresponding non-oxidized protein acceptor.

Cholesterol loading enhances the apolipoprotein-mediated efflux of cholesterol and phospholipids (Jian, B., de la Llera-Moya, M., Ji, Y., Wang, N., Phillips, M. C.,

Swaney, J. B., Tall, A. R., and Rothblat, G. H. (1998) *J. Biol. Chem.* **273,** 5599-5606). Fig. 5 shows that similar to cholesterol, the efflux of [^{3}H]-phospholipids from lipid-laden hMDM was greater for lipid-free apoA-I$_{+32}$ than apoA-I.

In addition to cholesterol and phospholipids, α-TOH is another important constituent of all cell membranes. We therefore assessed whether apoA-I$_{+32}$ is also more effective than apoA-I in removing this lipid from [^{14}C]-α-TOH-labeled hMDM. Incubation of such cells with lipid-free apolipoprotein A-I resulted in a time-dependent efflux of [^{14}C]-α-TOH. The extent of this efflux was greater for apoA-I$_{+32}$ than apoA-I (Fig. 6A). As α-TOH and cholesterol efflux experiments were performed under comparable conditions, the relative extents of release of the two lipids were compared. Cells acquired 224 741 ± 14 503 cpm per mg cell protein (mean ± SD, n = 3) or 4.9 % of the [^{14}C]- α-TOH added. 4.9 ± 0.2 and 6.9 ± 0.4 % of cell [^{14}C]- α-TOH was released to apoA-I and apoA-I$_{+32}$, respectively after 3 h. These values and the kinetic of the process were comparable to those of [^{3}H]-cholesterol (compare Figs. 3 & 5). Thus, apoA-Iremoves these two lipids with comparable efficacy from hMDM, although apoA-I$_{+32}$ is superior in doing this compared with apoA-I.

Example 4 – Oxidized Apolipoproteins in Human Aorta

Human aortas were collected, divided into different sections according to increasing degree of lesion severity, HDL isolated by sequential density ultra-centrifugation and its content of apoA-I and apoA-I$_{+32}$ assessed by HPLC as described above. Early developmental stages are referred to as stages I and II, whereas late developmental stages are referred to as stages III and IV, in accordance with the Stary Classification System:

Human aortas were obtained post-mortem within <24 hours of death of the donor, placed in saline and then brought to the laboratory on ice. Aortas were then rinsed in buffer A (10 mM phosphate buffer, pH 7.3 (50 mL) containing 150 mM NaCl, 0.3 mM EDTA, 100 μM diethylenetriamine pentaacetic acid, 50 μg/mL

soybean trypsin inhibitor, 100 μM BHT and 10 mM aminotriazole) and frozen at – 80 °C. Batches of 20 – 30 aortas were thawed and sections representing different developmental stages separated according to the Stary Classification (Stary et al. *Arterioscler. Thromb.* 14:840-856(1994)). After removal of surrounding adventitia and media, intimas of the various sections representing the four different developmental stages were rinsed again in buffer A, blotted dry, weighed and lesion HDL isolated principally as described by Leeuwenburgh et al. *J. Biol. Chem.* 272:3520-3526 (1997). Intima (~ 10 g) was then frozen in liquid nitrogen and pulverized using a ceramic mortar and pestle. The tissue powder was then reconstituted in buffer A by gentle rotation end-over-end for 12-16 hours at 4 °C and the resulting solution clarified by centrifugation at 5 000 x g for 15 min at 5 °C. The top (lipemic layer) and pellet were discarded, and the supernatant then subjected to sequential flotation ultra-centrifugation. The resulting lesion HDL was desalted and subjected to RP-HPLC as described above for plasma HDL.

It was found that the proportion of apoA-I present as apoA-I_{+32} was substantially greater in lesion than plasma HDL treated similarly. Furthermore, more apoA-I_{+32} was found in late stages of atherosclerosis compared to early stage ($P < 0.05$). This is shown in Figure 8 and in Table V.

Table V – Analysis of Aortic 'HDL" by RP-HPLC

	$A1_{+32}$ % of ($A1+A1_{+32}$)
Stage I, II	
M53	37.6
M56	38.6
M58	64.3
M59	47.8
M61	29.02
M62	54.3
M66	57.4
M2	13.9
M67	43.1
Mean	**42.9**
stdv	**15.4**
II-III, III, III-IV, IV	
M51	54.44
M52	93.05
M57	38.58
M60	47.84
M63	78.81
M64	62.27
M65	85.23
M68	52.84
Mean	**64.1**
stdv	**19.4**
all samples Mean	**52.9**
stdv	20.1

Example 5 – Measuring apoA-I_{+32} in patients with eNOS a/b genotype and other risk factors of coronary vascular disease.

Cord blood samples were collected post-partum. Blood was centrifuged within 10 minutes of collection and serum samples stored at –70 °C until HDL was isolated and analyzed for apoA-I and apoA-I_{+32} by HPLC as described above .

It was found that there is a significant correlation between eNOS genotype and percentage of HDL's apoA-I present as apoA-I_{+32}. HDL isolated from serum of the eNOSa/b genotype (16.6 +/-6.5%, n = 4) has 5 times higher apoA-I_{+32} than that of serum obtained from subjects with the common eNOSb/b genotype (3.3 +/- 0.9%, n = 8) (p = 0.016).

Similarly, HDL isolated from serum of tobacco smokers (14.1 +/- 4.8, n=5) also had a higher proportion of apoA-I_{+32} than HDL from serum of non-smokers (5.5 +/- 1.9%, n = 12) (p = 0.064).

Finally, it is understood that various modifications, alterations and/or additions may be made to the example specifically described and illustrated herein without departing from the spirit and scope of the invention.

Claims:

1. An oxidized apoA-I having at least a Met residue 86 oxidized to Met (O).

2. An oxidized apoA-I according to claim 1 further including a Met residue 112 oxidized to Met (O).

3. An oxidized apoA-I according to claim 1 or 2 which is 16 or 32 mass units heavier than native apoA-I.

4. An oxidized apoA-II comprising an apoA-II dimer and wherein said dimer is oxidized at a single or several Met residue(s).

5. An oxidized apoA-II according to claim 4 having at least one Met residue at position 26 oxidized to Met (O).

6. A reconstituted HDL comprising an oxidized apolipoprotein said apolipoprotein having at least one Met residue oxidized to Met (O).

7. A reconstituted HDL according to claim 6 wherein the apolipoprotein is apoA-I having at least a Met 86 residue oxidized to Met (O).

8. A reconstituted HDL according to claim 7 wherein both a Met 86 and Met 112 residue are oxidized to Met (O).

9. A reconstituted HDL according to claim 6 wherein the apolipoprotein is apoA-II comprising an apoA-II dimer and wherein at least one Met 26 residue of an apoA-II dimer is oxidized to Met (O).

10. A method of inducing an increased efflux of lipids from cells, said method comprising subjecting said cells to an oxidized apolipoprotein wherein said apolipoprotein is oxidized at a single or several Met residue(s).

11. A method according to claim 10 wherein said apolipoprotein is an apolipoprotein according to anyone of claims 1 to 5.

12. A method according to claim 10 or 11 wherein the apolipoprotein is apoA-I_{+32}.

13. A method according to claim 10 or 11 wherein the apolipoprotein is apoA-II_{+32}.

14. A method according to claim 12 wherein apoA-I_{+32} is selected from apoA-I_{+32} oxidized at Met 86 or apoA-I_{+32} oxidized at Met 86 and Met 112.

15. A method according to claim 13 wherein at least one Met 26 residue of apoA-II_{+32} is oxidized to Met (O).

16. A method of inducing an increased efflux of α-tocopherol (α-TOH) from cells, said method comprising subjecting said cells to an oxidized apolipoprotein oxidized at a single or several specific Met residue(s).

17. A method according to claim 16 wherein said apolipoprotein is an apolipoprotein according to anyone of claims 1 to 5.

18. A method of lowering lipid concentration in cells, said method comprising inducing an increased efflux of lipids from the cells by administering an effective amount of oxidized apolipoprotein oxidized at a single or several Met residue(s).

19. A method according to claim 18 wherein the apolipoprotein is an apolipoprotein according to any one of claims 1 to 5.

20. A method according to any one of claims 10 to 19 wherein said cells are monocyte derived macrophages.

21. A method of preventing or treating lipid- associated conditions wherein said condition is associated with high levels of lipids, said method comprising administering an effective amount of an apolipoprotein oxidized at a single or several Met residue(s).

22. A method according to claim 21 wherein the apolipoprotein is an apolipoprotein according to any one of claims 1 to 5.

23. A method according to claim 21 or 22 wherein the lipid associated condition is selected from the group including coronary vascular disease, ischemic heart disease, atherosclerosis, and dyslipidemias.

24. A method according to claim 23 wherein the lipid associated condition is atherosclerosis.

25. A method according to any one of claims 10 to 24 wherein said apolipoproteins are delivered as a reconstituted HDL according to any one of claims 6 to 9.

26. A method of assessing genotype, said method comprising detecting in a biological sample the presence of an oxidized apolipoprotein having an oxidation at a single or several Met residue(s) and comparing against a condition where the genotype is absent or different.

27. A method according to claim 26 wherein said genotype to be assessed is associated with an increased risk of coronary disease for detecting an increased risk of coronary disease.

28. A method according to claim 26 or 27 wherein the genotype to be assessed is a polymorphism of a genotype associated with an increased risk for coronary disease.

29. A method according to any one of claims 26 or 28 wherein the genotype associated with increased risk of cardiovascular disease is selected from the group including endothelial nitric oxide synthase (eNOS) genotype a/b, Asp_{298} variant of eNOS, or conditions resulting in high levels of circulating homocysteine.

30. A method according to claim 29 wherein the genotype is eNOS a/b genotype.

31. A method according to any one of claims 26 to 30 wherein the apolipoprotein is an apolipoprotein according to any one of claims 1 to 5.

32. A method of diagnosing a lipid associated condition wherein said condition is characterized by high lipid concentration and/or genetic factors known to be associated with an increased risk of cardiovascular disease, said method comprising determining levels of an apolipoprotein oxidized at a single or several Met residue(s) and comparing against levels in an absence of said lipid-associated condition.

33. A method according to claim 32 wherein the apolipoprotein is an apolipoprotein according to any one of claims 1 to 5.

34. A method according to claim 33 wherein the genotype is an eNOS a/b genotype.

35. A method of determining the extent of a lipid-associated condition wherein said extent of the lipid-associated condition is characterized by a high lipid and/or apolipoprotein concentration adjusted level of an oxidized apolipoprotein having an oxidation at a single or several Met residue(s) and comparing against an absence of the lipid-associated condition.

36. A method according to claim 35 wherein the apolipoprotein is according to any one of claims 1 to 5.

37. A method according to claim 35 or 36 wherein the lipid-associated condition is selected from the group including coronary vascular disease, ischemic heart disease, atherosclerosis, and dyslipidemias.

38. A method according to claim 37 wherein the lipid-associated condition is atherosclerosis.

39. A method of measuring oxidative stress *in vivo* said method comprising detecting the presence of an oxidized apolipoprotein having an oxidation at a single or several Met residue(s) and comparing against a condition in the absence of oxidative stress.

40. A method according to claim 39 wherein the apolipoprotein is according to any one of claims 1 to 5.

41. A method according to any one of claims 26 to 39 wherein the oxidized apolipoprotein is detected by any of the methods selected from the group including HPLC, FPLC, or methods using ligands/agents which bind to the oxidized forms of apoA-I or apoA-II, including antibodies to the oxidized forms of apoA-I or apoA-II.

42. A method of collecting biological samples for determination of oxidized apolipoproteins, said method comprising the steps of:

obtaining a biological samples; and

subjecting the sample to conditions which reduce further oxidation of a single or several methionine residue(s) of the apolipoprotein.

43. A method according to claim 42 wherein the conditions which reduce further oxidation of a single or several methionine residue(s) of the apolipoprotein include storing a sample at –70 °C within approximately 10 minutes of collection.

44. A method according to claims 42 and 43 wherein the conditions include treating the sample so as to dissassociate apolipoproteins from lipids.

45. A method according to claim 44 including treating the sample with sodium dodecyl sulphate.

46. A method according to any one of claims 42 to 45 wherein the sample is selected from the group including plasma, serum, blood, interstitial fluid, blood vessel(s), saliva or any other tissue.

Figure 1

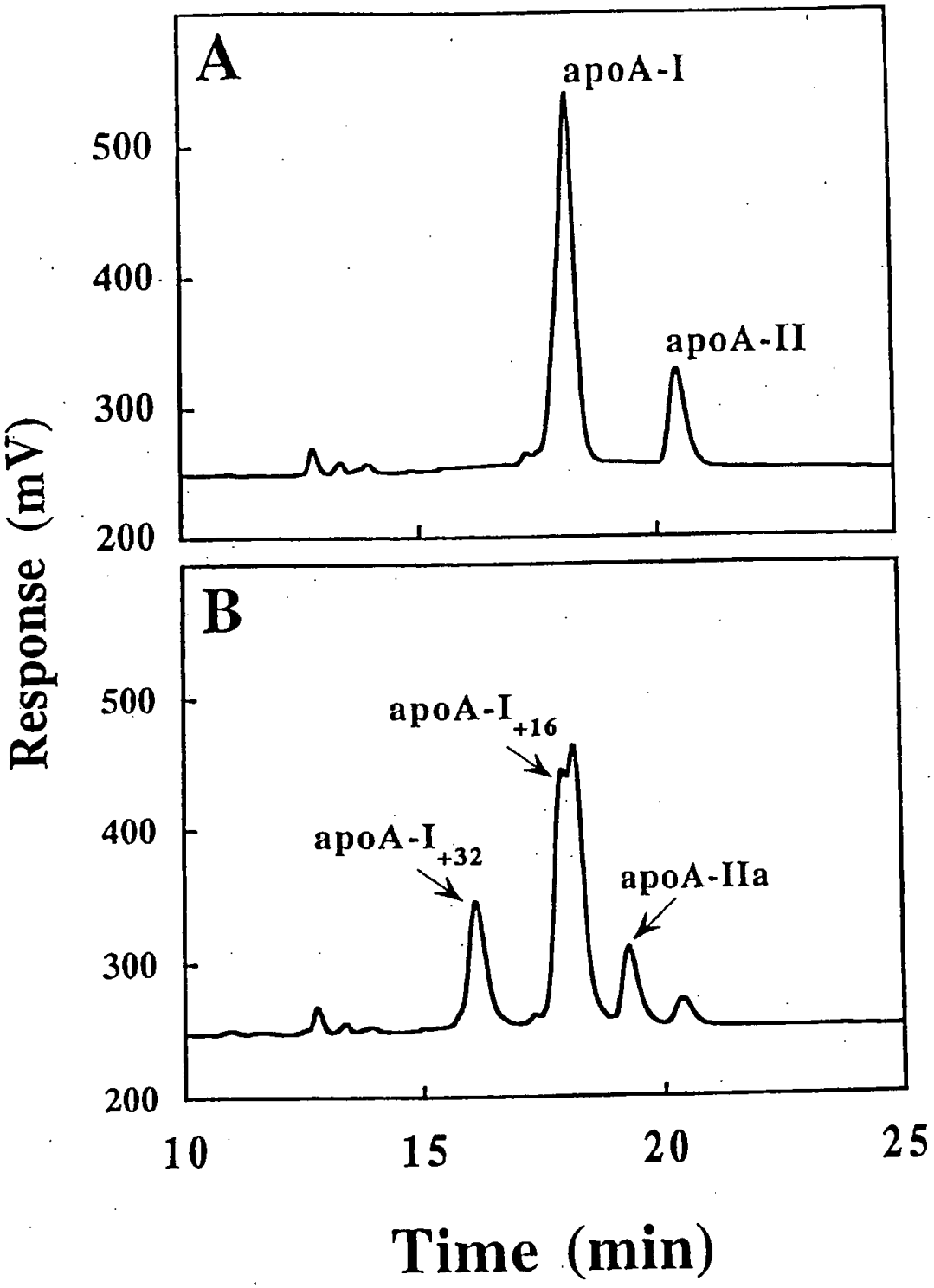

A
apoA-I
apoA-II
500
400
300
200
B
apoA-I+16
apoA-I+32
apoA-IIa
500
400
300
200
Response (mV)
10
15
20
25
Time (min)

Figure 2

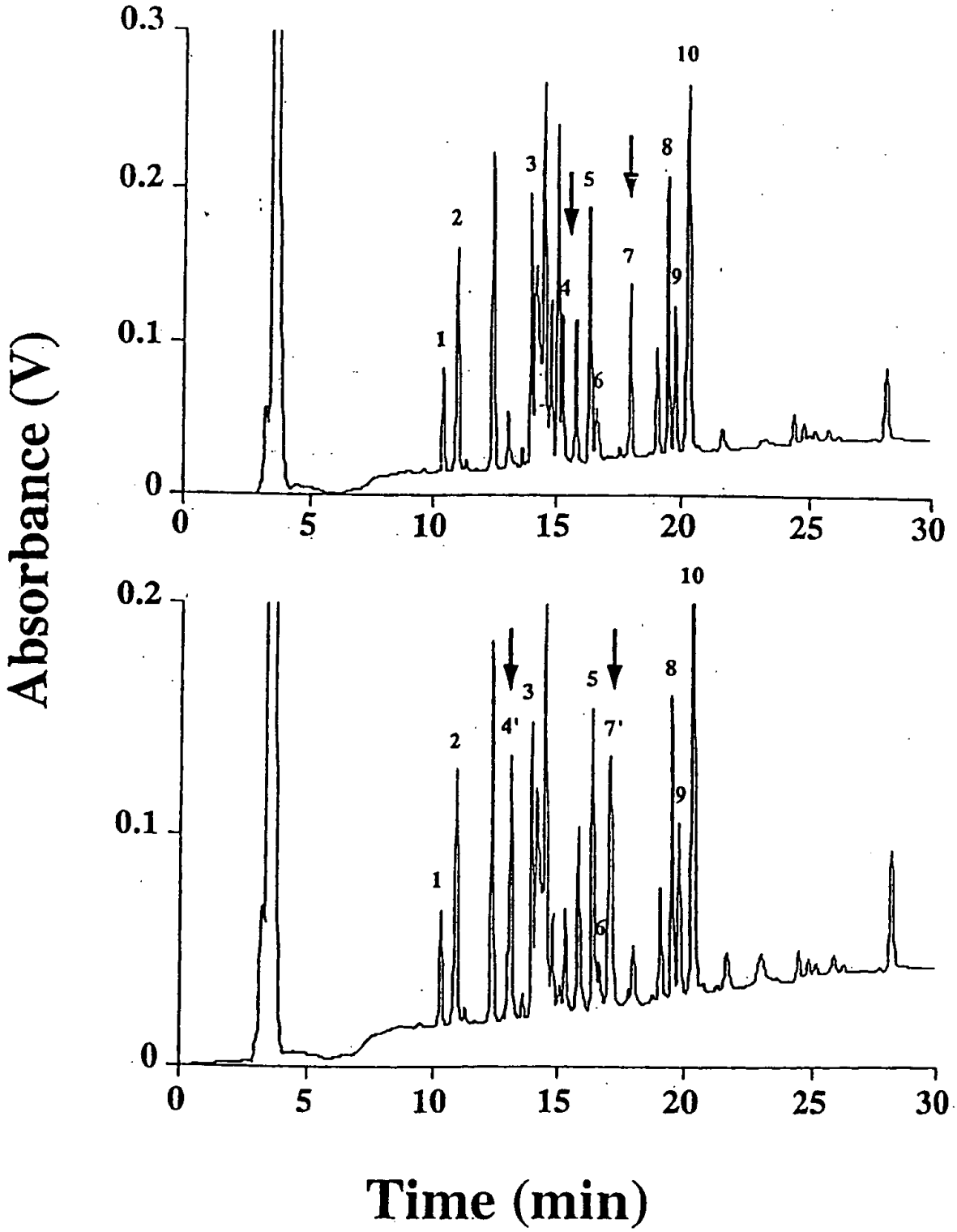

Absorbance (V)
0.3
0.2
0.1
0
0
5
10
15
20
25
30
1
2
3
4
5
6
7
8
9
10
0.2
0.1
0
1
2
3
4'
5
6
7'
8
9
10
Time (min)

Figure 3

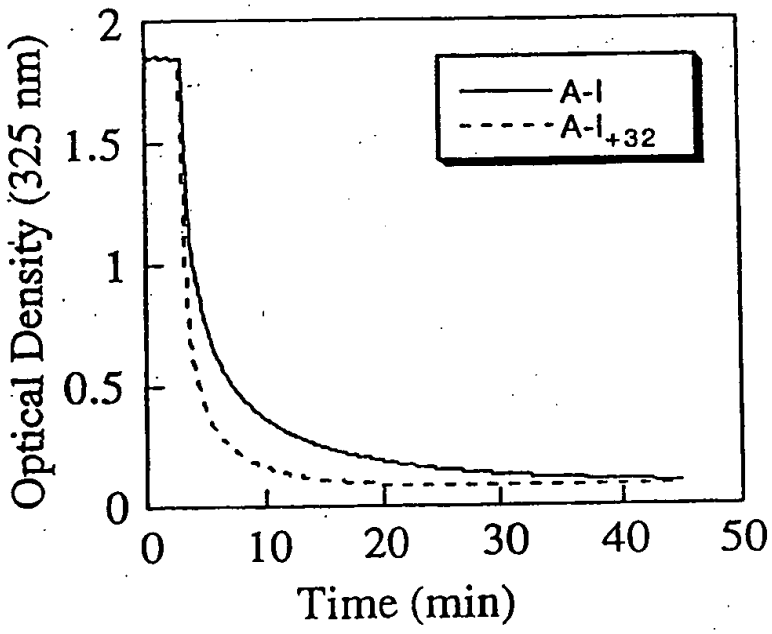

Optical Density (325 nm)
2
1.5
1
0.5
0
0
10
20
30
40
50
Time (min)
A-I
A-I+32

Figure 4

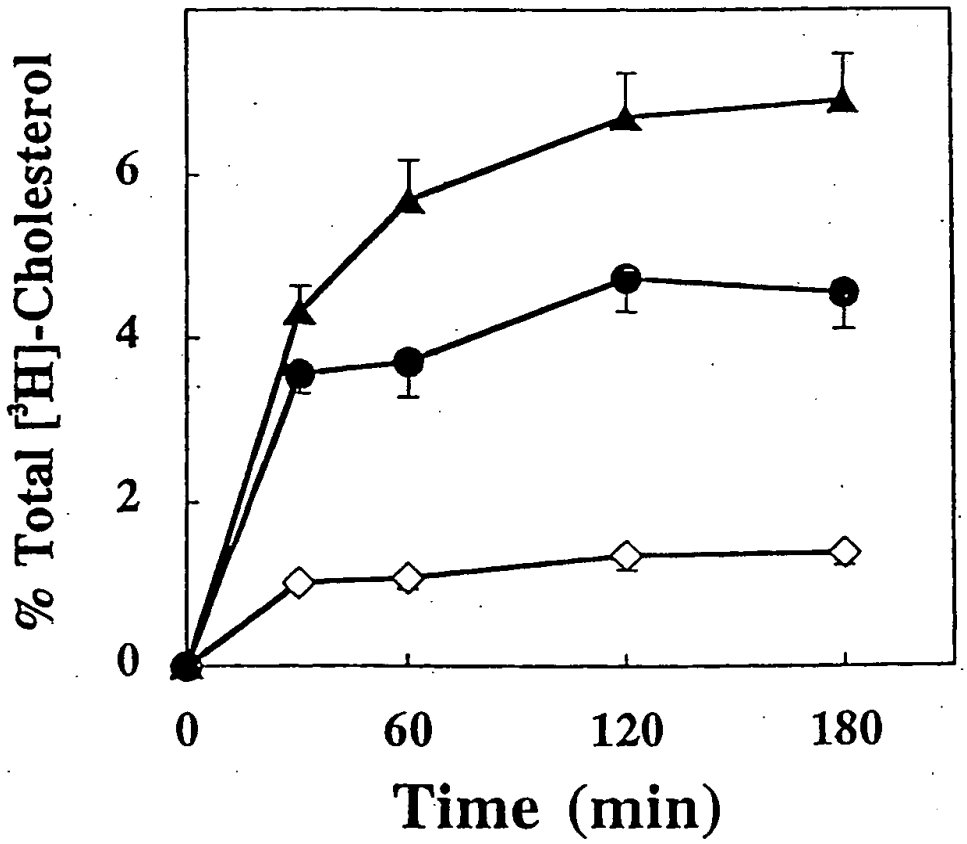


Figure 5

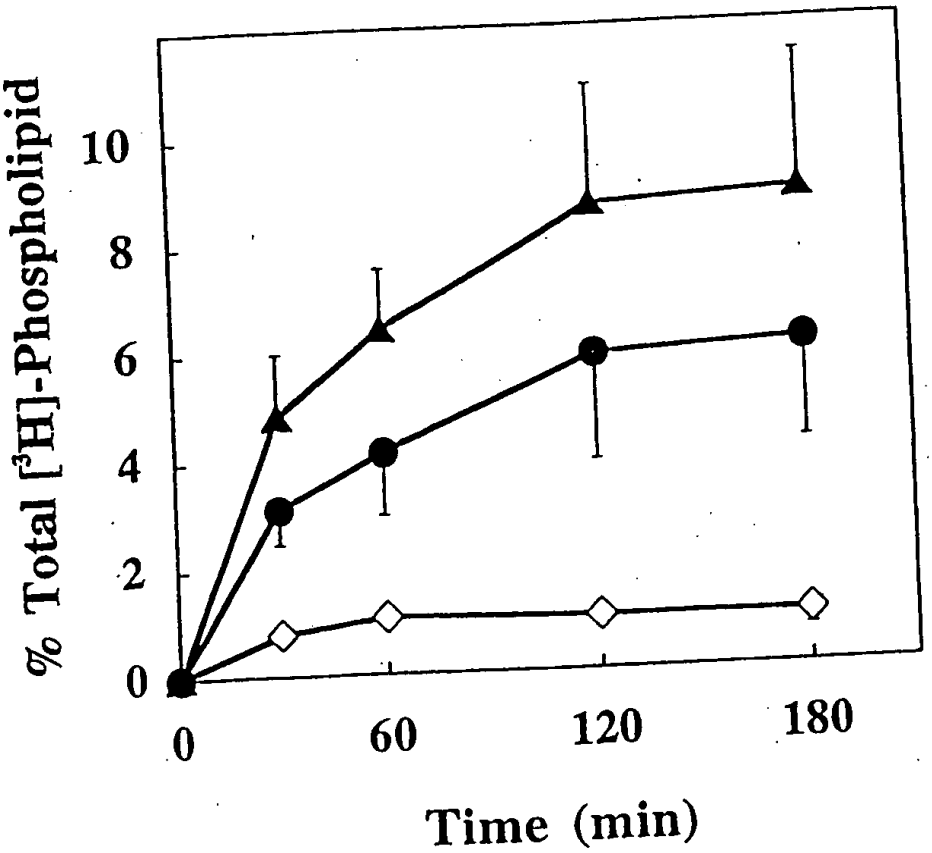


Figure 6

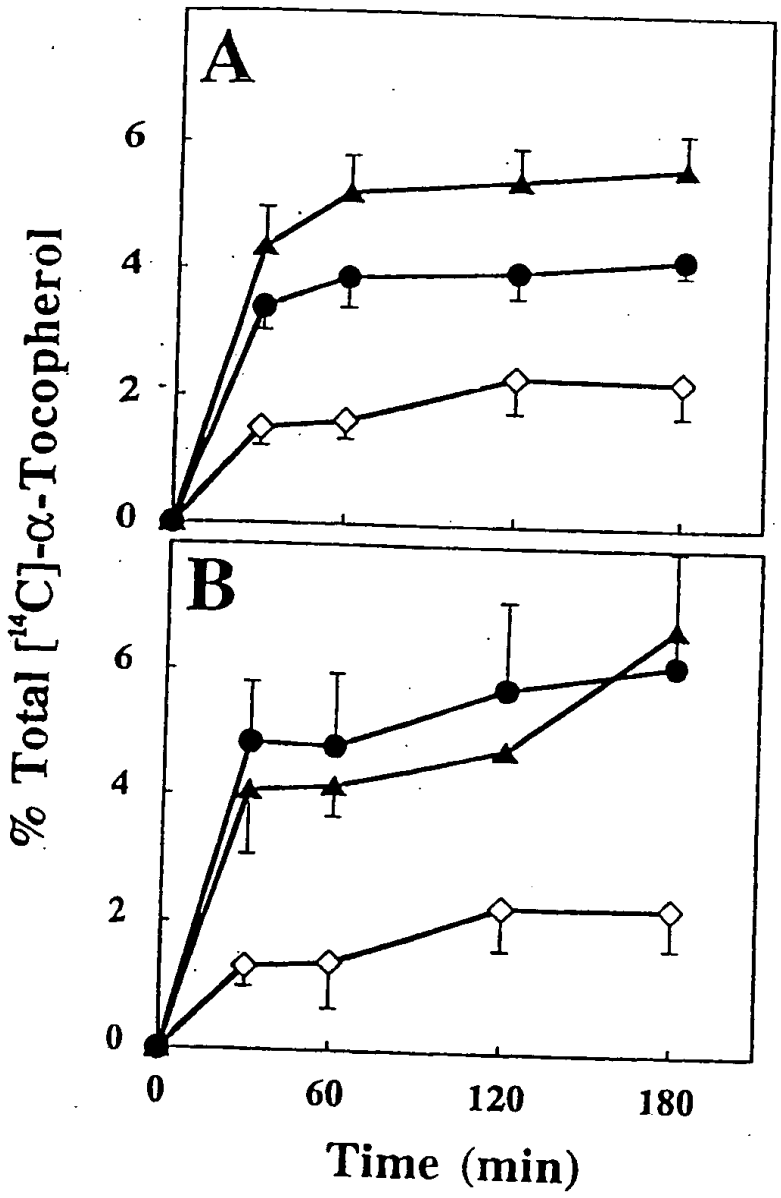
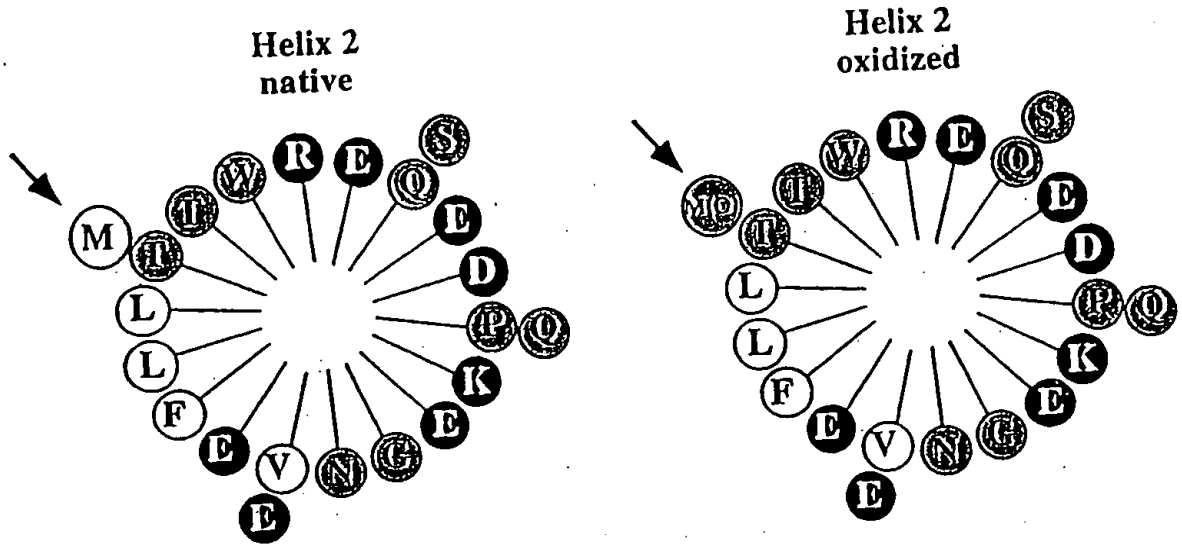
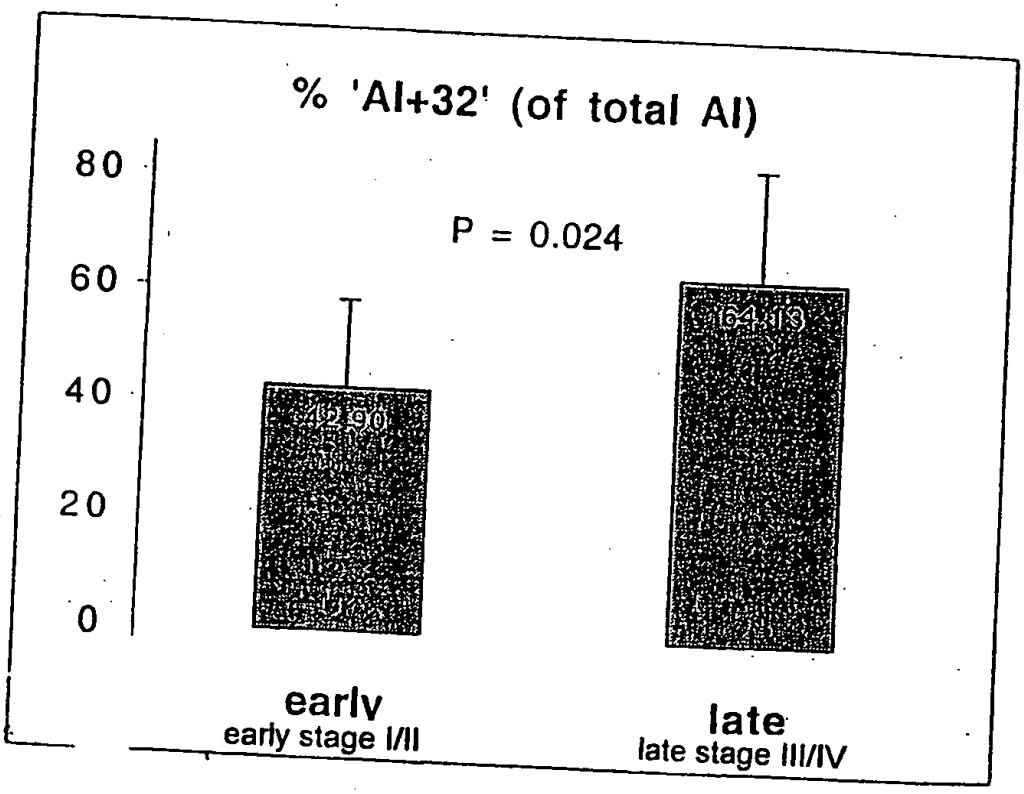


Figure 7



Helix (residues)	Hydrophobic moment (kcal/mol)	
	native	oxidized
2 (66-87)	29.7	25.7
4 (99-120)	24.8	22.0
6 (143-164)	21.7	16.5

Figure 8


% 'AI+32' (of total AI)
80
60
40
20
0
P = 0.024
42.90
64.13
early
early stage I/II
late
late stage III/IV

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01463

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C07K 14/775, 16/18; A61K 38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, DERWENT: Keywords - based on apolipoprotein, oxidise, methionine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	The Journal of Biological Chemistry, Vol. 273, No. 11, 13 March 1998, pp 6088-6095; B. Garner et al.: "Oxidation of High Density Lipoproteins. II Evidence ..." See particularly p 6090, 6093-5	4-6, 9-13, 15-46
X	The Journal of Biological Chemistry, Vol. 273, No. 11, 13 March 1998, pp 6080-6087; B. Garner et al.: "Oxidation of High Density Lipoproteins. I Formation ..." See particularly p 6083, 6085-6	4-6, 9-13, 15-46
X	Biochemistry, 17 June 1997, 36 (24) pp 7615-24; L. M. Roberts et al.: "Structural Analysis of Apolipoprotein A-I: Limited Proteolysis of Methionine-Reduced and -Oxidized ..." See particularly p 7618-19, 7621-23	6, 10-11, 16-46

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
8 February 2001

Date of mailing of the international search report
16 February 2001

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01463

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Lipid Research, March 1988, Volume 29 (3), pp 309-18; G. M. Anantharamaiah et al.: "Effect of oxidation on the properties of apolipoproteins A-I and A-II" See particularly p 312-313, 315-318	4-6, 9-13, 15-46
P, X	The Journal of Biological Chemistry, Vol. 275, No. 26, 30 June 2000, pp 19536-19544; U. Panzenböck et al.: "Oxidation of Methionine Residues to Methionine Sulfoxides Does Not Decrease Potential Antiatherogenic Properties of Apolipoprotein A-I". See whole document	1-46
P, X	Biochemical Journal, 1 March 2000, 346, Pt 2, pp 345-354; C. Bergt et al.: "Reagent or myeloperoxidase-generated hypochlorite affects discrete regions in lipid-free and lipid-associated human apolipoprotein A-I". See whole document	1-46
X	Journal of Lipid Research, April 1999,Vol. 40 (4), pp 686-98; C. Yang et al.: "Selective modification of apoB-100 in the oxidation of low density lipoproteins by myeloperoxidase in vitro". See whole document	10, 16, 18, 20, 21, 23, 24, 26-30, 32, 35-39, 41-46
X	Biochemical Journal, 1 May 1999, 339 (Pt 3), pp 489-495; L.J. Hazell et al.: "Secondary radicals derived from chloramines of apolipoprotein B-100 contribute to HOCl-induced lipid peroxidation of low-density lipoproteins". See whole document	10, 16, 18, 20, 21, 23, 24, 26-30, 32, 35-39, 41-46
X	Free Radical Biology & Medicine, 1997, Vol. 23, No. 1, pp 82-89; C. Yang et al.: "Oxidative modifications of ApoB-100 by exposure of low density lipoproteins to HOCl in vitro". See whole document	10, 16, 18, 20, 21, 23, 24, 26-30, 32, 35-39, 41-46
A	US 5,900,359 A (Matsuura et al.) 4 May 1999 See whole document	26-46
A	Journal of Lipid Research, September 1991, Vol. 32 (9), pp 1465-76; A. von Eckardstein et al.: "Site-specific methionine sulfoxide formation is the structural basis of chromatographic heterogeneity of apolipoproteins A-I, C-II, and C-III". See whole document	1-46

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU00/01463

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	5900359	AU	77072/94	EP	722086	WO	9509363

END OF ANNEX